Growth, metabolism and immune responses to evisceration and the regeneration of viscera in sea cucumber, *Apostichopus japonicus*

Yuanqi Zang, Xiangli Tian, Shuanglin Dong, Yunwei Dong

Apostichopus japonicus

Abstract

Growth, metabolism and immune responses of sea cucumber, *Apostichopus japonicus*, following evisceration induced artificially, were investigated in this study. The body weight, specific growth rate (SGR), oxygen consumption rate (OCR), activities of immunoenzymes including acidic phosphatase (ACP), alkaline phosphatase (AKP) from hydrolytic system, and catalase (CAT), superoxide dismutase (SOD), total antioxidant capacity (T-AOC) and malonyl-dialdehyde (MDA) from antioxidant system in muscle, intestine and respiratory tree were measured to evaluate the physiological responses of *A. japonicus* to evisceration and regeneration of viscera. The results showed that body weight of sea cucumber significantly dropped following evisceration and then increased gradually with digestive function resumed. Accelerated growth rates were observed in the regeneration group from the 10th day to 20th day, but there was no significant difference between the regeneration group and control group at the end of the experiment. OCR reduced rapidly after evisceration and increased gradually afterwards. Compared to the control, no significant difference in OCR was found on the 45th day (*P* > 0.05). The immune responses of *A. japonicus* were highly tissue-specific during the regeneration of viscera. Both ACP and AKP activities in muscle peaked on the 10th day after evisceration, then decreased gradually to normal on the 45th day (*P* > 0.05), while those in the regenerated tissues exhibited a differential rising trend from the 20th day after evisceration. SOD activity in muscle was not significantly influenced by evisceration; however, in the regenerated tissues it was enhanced and significantly higher than those in the control on the 45th day (*P* < 0.01). CAT activity in muscle of the regeneration group was significantly higher than in the control during the experiment (*P* < 0.05). Comparatively, CAT activity in the respiration tree increased invariably after evisceration, while in the intestine CAT activity was not significantly affected by evisceration (*P* > 0.05). Significant increase was found in both T-AOC and MDA in all three tissues after evisceration (*P* < 0.05); however, they all returned to normal at the end of the experiment. Results of this study indicated that growth, metabolism and immune responses including hydrolytic and antioxidant enzymes in sea cucumber were significantly influenced by evisceration. Although the regeneration group did not catch up with the control in body weight, SGR, OCR, major non-specific immune parameters resumed to normal level within 45 days after evisceration, indicating that *A. japonicus* might have recovered physiologically from evisceration. Results from this study first presented comprehensive and valuable data of physiological responses to evisceration and the regeneration of viscera in *A. japonicus*, which would have important implications for those farmers engaged in sea cucumber culture.

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1. Introduction

A phenomenon known in the representatives of holothurians such as *Apostichopus japonicus* (Sun et al., 2011; Tan et al., 2008; Zheng et al., 2006), *Holothuria glaberrima* (Mashanov et al., 2012; Quiñones et al., 2002; Suárez-Castillo et al., 2004), etc., is spontaneous rejection of internal organs, i.e., evisceration (Shukalyuk and Dolmatov, 2001). Evisceration has been demonstrated in most cases as a response to artificial stimuli with fewer cases of its definite occurrence in natural conditions such as body injury, overcrowding or water pollution (Wilkie, 2001), and it has been recognized that regeneration occurs after these cases (Emson and Wilkie, 1980; Swan, 1961).

A wealth of information has been accumulated on the regeneration of tissues in several species of holothurians. Bertolini (1930) explored the cellular events that enable regeneration of the digestive tract and first accurately analyzed histological aspects of the regenerative process in *Stichopus regalis*. Dawbin (1949) studied the regeneration of viscera in *Stichopus mollis* and Kille (1942) focused on the regeneration of reproductive system in *Holothuria parvula*. Over the last decade, more studies had been conducted to investigate the histological and...
cellular aspects, including cell division, dedifferentiation, and migration within the intestine, body wall and other appendages (Dolmatov and Gininova, 2009; García-Arrarás et al., 2006; Murray and García-Arrarás, 2004; Sun and Zheng, 2005; Wang and Li, 2007; Zheng et al., 2006). More recently, the molecular events involved in regeneration have also started to be uncovered (Mashanov et al., 2010, 2012; Sun et al., 2011). Particularly, an up-regulation expression of Wnt9, TCTP, and Bmp1/Tll in visceral regeneration of the sea cucumber *H. glaberrima* was found, and it's the first attempt to map the expression domains of Wnt9 in a species outside the vertebrate lineage (Mashanov et al., 2012). However, to our knowledge, the responses of growth, metabolism and immune following the evisceration in holothurian species have not been fully studied until now.

The immune response in echinoderms includes the recognition of foreign materials (non-self), expulsion of non-self or rendering it innocuously, and wound healing (Nusetti et al., 2005; Ramírez-Gómez et al., 2008). These defense mechanisms are conducted by cellular and humoral immune responses, in which immune enzymes are commonly involved as non-specific immunity. As hydrolytic enzymes, ACP and AKP participate in the degradation of foreign proteins, carbohydrates and lipids (Ottaviani, 1984; Pipe et al., 1993; Xue and Renault, 2000). Antioxidant system is important because reactive oxygen species (ROS) are continuously generated by a variety of cellular processes, such as superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (OH), and singlet oxygen (Roch, 1999). Antioxidant defenses can be measured by total antioxidant capacity (T-AOC). The key enzymatic players include superoxide dismutase (SOD) and catalase (CAT), which detoxify O$_2^-$ and H$_2$O$_2$, respectively (Hermes-Lima et al., 1998). Terminal products including MDA formed during the lipid peroxidation process which was induced by ROS, are very active and capable of, cross-linking of membrane proteins containing amino groups (Kehrer, 1993).

The sea cucumber, *A. japonicus* Selenka, is one of the most important holothurian species cultured in China (Liao, 1980). In 2010 total production of the sea cucumbers was $1.30 \times 10^4$ t, within $15.01 \times 10^4$ ha (Fisheries Bureau, Agriculture Department, China, 2011). However, average yield of *A. japonicus* was still very low ($868.10$ kg/ha or so in 2010), due to limited knowledge of the species’ eco-physiology. As same as other holothurian species, severe evisceration induced by various natural and artificial factors commonly occurs during transport and culture for *A. japonicus*, causing higher mortality and lower yield (Zheng et al., 2006). After expulsion of the internal organs, the growth of sea cucumbers drops sharply, and due to loss of the respiratory tree, the body wall will perform respiration for the whole animal (Choe, 1963). This could affect metabolism, as well as immune functions for different tissues during the period of the regeneration of internal organ (Tan et al., 2008). Regeneration of viscera in *A. japonicus* had significant effects on oxygen consumption rate and ammonia excretion, as well as body consumption and energy content, as Tan et al. (2008) described.

### Table 1

<table>
<thead>
<tr>
<th>Moisture (%)</th>
<th>Protein (%)</th>
<th>Dry matter (%)</th>
<th>Lipid (%)</th>
<th>Energy (kJ/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.31 ± 0.5</td>
<td>17.38 ± 0.2</td>
<td>22.89 ± 0.2</td>
<td>1.67 ± 0.1</td>
<td>12.51 ± 0.1</td>
</tr>
</tbody>
</table>

Values were given as mean ± SE. Protein, lipid and dry matter were measured as percentages on a dry matter basis.

The study was designed to investigate the growth, metabolism and immune status to evisceration and the regeneration of viscera in *A. japonicus*. Activities of acidic phosphatase (ACP), alkaline phosphatase (AKP), catalase (CAT), superoxide dismutase (SOD), total antioxidant capacity (T-AOC), and malonyl-dialdehyde (MDA) in muscles, intestines and respiratory trees were monitored as biomarkers of the regeneration.

### 2. Material and methods

#### 2.1. Experimental animals and acclimation

Sea cucumbers were collected in May 2009 from the coastal culture pond located in Jimo, Qingdao City, Shandong Province, P. R. China. The animals were cultured in a 1000-L fiberglass tank filled with filtered seawater for 15 days to fully ensure thermal and environmental adaptation. During acclimation, the seawater replenished daily and maintained at $17 ^\circ C \pm 0.5 ^\circ C$ and 28 to 30% salinity. The animals were fed to satiation once daily at 18:00 with a formulated feed (*Sargassum* spp., fish meal, sea mud, wheat, vitamin and mineral pre-mixes; $9.31 \pm 0.5$% moisture, $17.38 \pm 0.2$% protein, $22.89 \pm 0.2$% dry matter, $1.67 \pm 0.1$% lipid, $12.51 \pm 0.1$ kJ g$^{-1}$ energy; Table 1). The experiments were performed between 17 May 2009 and 30 June 2009.

![Fig. 1. Changes in body weight of *A. japonicus* during the regeneration of viscera. Each bar represents means ± SE. Asterisks indicate significant differences with respect to control values: *P*<0.05; **P*<0.01.](image-url)
2.2. Experimental design and management

After acclimation, 200 sea cucumbers of similar size (51.03 ± 2.15 g) were arbitrarily collected and equally divided into the regeneration and control groups. 100 sea cucumbers as one group were induced evisceration by injecting KCl 0.35 mol L\(^{-1}\) (2% body weight) into the coelom (Tan et al., 2008). One or two minutes later, sea cucumbers eviscerated and were randomly distributed into 20 rectangular glass aquaria (55×30×35 cm, water volume of 45 L) with 5 individuals per aquarium (regeneration group). The other 100 sea cucumbers, the control group, were not eviscerated. They were randomly distributed into 20 aquaria with 5 individuals per aquarium as well.

Sea cucumbers in the regeneration group and the control were cultured with normal feed supply (feed over 5% of wet body weight) once a day (18:00 h) in aquaria during the period of the experiment. Uneaten food in the tank was collected 24 h after feeding by pipetting. Feces were collected twice daily by pipetting before feeding.

Seawater used in the experiment was filtered by a composite sand filter, and 50% of the water in each aquarium was exchanged daily to ensure good water quality. Aeration was provided continuously to maintain dissolved oxygen above 6 mg L\(^{-1}\).

During the experiment, seawater temperature and salinity were controlled at 17 ± 0.5 °C and 28–30‰ respectively. The ammonia was less than 0.025 mg L\(^{-1}\) and pH ranged from 7.8 to 8.2. A simulated natural photoperiod (14 h light:10 h dark) was used throughout the period of experiment.

2.3. Measurement of growth

Sea cucumbers in 5 aquaria for each treatment were assigned to measure the growth rate of animals. Sea cucumbers were weighed every 5 days during the experiment. Before weighing, the animals were fasted for 24 h to evacuate their guts, and then weighed as previously described (Zhou et al., 2006).

2.4. Determination of oxygen consumption rate

Sea cucumbers in 5 aquaria for each treatment were used for the oxygen consumption rate determination (OCR). OCR was determined every 5 days for the regeneration and control groups during the experiment. Prior to the determination of oxygen consumption, sea cucumbers were starved for 24 h to reduce any associated metabolic responses. In the regeneration and control groups, there were five replicates and one blank control to correct for the respiration of bacteria in the water. The tested animals were put individually into a 1 L conical flask. After a 4 h test period, the final level of dissolved oxygen in each chamber was determined using the Winkler method (Ji et al., 2008; Strickland and Parsons, 1968). Oxygen consumption rate (OCR) of sea cucumber was calculated with the following equation (Omori and Ikeda, 1984):

\[
OCR = \frac{(D_t V_t - D_0 V_0)}{W T}
\]

where, \(T\), time duration (h); \(D_t\), changes of the oxygen content (\(\mu g O_2\) L\(^{-1}\)) before and after test in the test bottles; \(D_0\), changes of the oxygen content (\(\mu g O_2\) L\(^{-1}\)) before and after test in the blank bottles; \(V_t\), volumes of the test bottles (L); \(V_0\), volumes of the blank bottles (L); \(W\), wet weight of the sea cucumber (g), determined as previously described (Zhou et al., 2006).

Regeneration procedure was considered finished when there was no significant difference of OCR between the regeneration group and the control group (Tan et al., 2008).

2.5. Tissue preparation and immune parameters assays

Sea cucumbers in 10 aquaria for each treatment were assigned to sample for immune parameters assays. Muscle, intestine and respiratory tree samples were collected every 5 days, and five animals were randomly sampled from 10 aquaria during the experiment. Muscle was removed from the posterior of the body. The whole intestine was removed by an incision at the esophagus and cloaca. It was then cut longitudinally and washed thoroughly in ice-cold physiological...
After rinsing, the three tissues were blotted dry with filter paper, and each sample was frozen with liquid nitrogen in an Eppendorf tube (1.5 mL) and stored at −80 °C until analyzed. Muscle samples were collected throughout the experiment. Samples of intestine and respiratory tree in the regeneration group were collected from the 20th day after evisceration due to unavailability of samples before then.

At each sampling interval, samples were thawed, weighed and homogenized in 5 volumes of ice-cold physiological saline using a manual glass homogenizer. The homogenates were then centrifuged at 10,000 g for 20 min at 4 °C. The supernatant was then pipetted into clean centrifuge vials and stored at 4 °C until analyzed (less than 12 h).

Protein content of the homogenates was measured following the method of Spector (1978), using bovine serum albumin as the standard. All assays for enzyme activities were carried out in duplicate and measured in the cuvette of a UV 2102PC spectrophotometer (Unico, Shanghai, China).

Acid and alkaline phosphatase (ACP and AKP) activity assays were carried out according to Barrett’s (1972) method using a commercial kit (Nanjing Jiancheng Biotech Company, China). Concentration of phenol was measured spectrophotometrically at 520 nm after incubation at 37 °C for 30 min (for ACP), or 15 min (for AKP). ACP and AKP activity was defined as the amount of phenol (mol) produced per milligram protein.

Superoxide dismutase (SOD) activity was determined according to Ji (1991) with an assay kit (Nanjing Jiancheng Biotech Company, China). Assay conditions were 65 μmol phosphate buffer, pH 7.8, 1 μmol hydrochloric hydroxylamine, 0.75 μmol xanthine and 2.3×10⁻³ IU xanthine dismutase. 50 μL of the supernatant given no blank reaction were incubated in the system for 40 min at 37 °C, and terminated with 2 mL 3.3 g L⁻¹ p-aminobenzene sulfonic acid and 10 g L⁻¹ naphthylamine. An SOD unit is defined as the amount of enzyme that inhibits the superoxide-induced oxidation (monitored at 550 nm) by 50%.

Catalase (CAT) activity was measured according to Göth (1991) with an assay kit (Nanjing Jiancheng Biotech Company, China). The base system including 4.60 μmol phosphate buffers, pH 7.4, 6.5×10⁻³ μmol H₂O₂ was incubated 1 min at 37 °C. The reaction was terminated immediately by 32.4 μmol ammonium molybdate. A CAT unit is defined as catalyzing the use of 1 μmol H₂O₂ per second.

Malondialdehyde (MDA) concentration was determined spectrophotometrically using the thiobarbituric acid method (Esterbauer and Cheeseman, 1990) with a commercial kit (Nanjing Jiancheng Biotech Company, China). The supernatant was added to an equal volume of 1% thiobarbituric acid in a 90 °C water bath for 10 min. After cooling, thiobarbituric acid reactive substance (TBARS) levels were estimated at 532 nm against a blank consisting of 5% cold trichloroacetic acid mixed with 1% thiobarbituric acid. Malondialdehyde bis acetal was used as a standard. Concentrations of lipid peroxidation compounds were expressed as nmol mg⁻¹ protein.

The total antioxidant capacity (T-AOC) was measured by the colorimetric technique as described by Miller et al. (1993) using a T-AOC commercial kit (Nanjing Jiancheng Biotech Company, China). These antioxidants reduce ferric ion (Fe³⁺) to ferrous ion (Fe²⁺).
The latter combines with phenanthroline and produces a stable chelate, which can be measured spectrophotographically at 520 nm. The T-AOC was determined in units per mg of tissue protein.

2.6. Statistical analysis

Statistical analysis of data was performed with a statistical package (SPSS 11.0 for windows, SPSS Inc., Richmond, CA, USA) Values were presented as means± standard error of the mean. Data for the body weight, SGR, OCR and immune parameters were tested for homogeneity of variances, then possible differences were compared among time intervals with one-way ANOVA followed by a Tukey’s HSD multiple comparison test Possible differences between the treatment and the control were tested by Student’s t-test. Differences were considered significant or extremely significant among treatment groups at a probability level of \( P<0.05 \) or \( P<0.01 \), respectively.

3. Results

3.1. Growth

Body weight of *A. japonicus* was significantly affected by evisceration (Fig. 1). Compared to the control group, body weight of animals dropped sharply following evisceration and continued to decrease until the 10th day, then increased gradually as viscera regenerated. However, body weight of the regeneration group was still significantly lower than the control group at the end of the experiment (\( P<0.01 \)).

The specific growth rate (SGR) of *A. japonicus* in regeneration group was much lower than the control by the 10th day after evisceration (\( P<0.01 \)), then accelerated growth rates were observed from the 10th day to 20th day. This trend, however, dampened after the 20th day. No significant difference was found between the regeneration group and the control from the 35th day to the end (\( P>0.05 \), Fig. 2).

3.2. Oxygen consumption rate

Oxygen consumption rates of *A. japonicus* were significantly affected by evisceration (Fig. 3). After evisceration, OCR of animals dropped sharply, continue to decrease until the 5th day (2.50±0.43 μg O₂·g⁻¹·h⁻¹) and then increased gradually until the 45th day (6.49±1.05 μg O₂·g⁻¹·h⁻¹). No significant difference was found between the regeneration group and the control at the end of the experiment (\( P>0.05 \)).

3.3. Immune parameters

Immune responses in muscle of *A. japonicus* were detected from the 1st day following evisceration to the end of the experiment, while those in intestine and respiratory tree were determined from Fig. 5. Changes of AKP activity in muscle, intestine and respiratory tree of *A. japonicus* during the regeneration of viscera. Each bar represents means±SE. Asterisks indicate significant differences with respect to control values: *\( P<0.05 \); **\( P<0.01 \), and NS indicates no significant difference.
20th day after evisceration when those tissues regenerated sufficiently for detection.

3.3.1. Acidic phosphatase activities

ACP activity in different tissues of *A. japonicus* in the control was highly tissue-specific and ranged from 10.20 ± 0.82 to 14.63 ± 0.64 U/g prot, 52.81 ± 2.31 to 583.85 ± 0.88 U/g prot and 71.96 ± 2.31 to 90.33 ± 2.01 U/g prot in muscle, intestine and respiratory tree, respectively (Fig. 4). ACP activity in the respiratory tree tissue was significantly higher than in intestine tissue, and muscle tissue had the lowest ACP activity (*P* < 0.05). With evisceration, ACP activity in muscle highly significantly increased compared to the control (*P* < 0.01). The maximum value occurred on the 10th day (22.41 ± 0.431 U/g prot), then it decreased gradually until the 45th day. No significant difference was found at the end of experiment (*P* > 0.05); however, for the regenerated tissues, ACP activity in both intestine and respiratory tree exhibited a rising trend totally from the 20th day after evisceration. ACP activity in intestine was significantly higher than in the control from the 30th day to the end of the experiment (*P* < 0.05), while that in the respiratory tree was significantly lower than in the control except on the 45th day (72.03 ± 3.06 U/g prot, *P* > 0.05).

3.3.2. Alkaline phosphatase activities

Values of AKP activity in muscle, intestine and respiratory tree for the control ranged from 11.40 ± 0.75 to 20.49 ± 1.21 U/g prot, 88.26 ± 3.20 to 104.08 ± 2.81 U/g prot and 33.07 ± 0.78 to 37.35 ± 0.94 U/g prot, respectively (Fig. 5). AKP activity in intestine of *A. japonicus* was the highest among the three different tissues, and that in respiratory tree was significantly higher than in muscle (*P* < 0.05). Compared to the control, AKP activity in muscle of the regeneration group significantly increased after evisceration, and the maximum value (39.43 ± 0.75 U/g prot) occurred on the 10th day. Furthermore, AKP activity decreased gradually, and no significant difference was found after the 30th day. AKP activity was significantly lower in the regenerated intestine than in the control on the 20th day after evisceration (*P* < 0.01), then increased gradually. No significant difference was found between the regeneration group and the control group at the end of experiment (*P* > 0.05). In the regenerated respiratory tree, AKP activity fluctuated from the 20th day after evisceration. However, no significant difference was found between the regeneration group and the control group throughout the experiment (*P* > 0.05).

3.3.3. Superoxide dismutase activities

SOD activity in muscle of *A. japonicus* fluctuated after evisceration (Fig. 6). During the regeneration of the viscera, there was no significant difference between the regeneration group and the control group (*P* > 0.05). For both regenerated intestine and respiratory tree, SOD activities were significantly higher than in the control (*P* < 0.01) on the 20th day after evisceration, and decreased gradually thereafter.
However, they were still significantly higher than those in the control at the end of experiment \((P<0.01)\).

3.3.4. Catalase activities

CAT activity in different tissues of \(A. japonicus\) in the control is tissue-specific, which ranged from 1.32 ± 0.09 to 1.72 ± 0.12 U/mg prot, 9.32 ± 0.08 to 9.61 ± 0.10 U/mg prot and 15.76 ± 0.97 to 17.16 ± 0.61 U/mg prot in muscle, intestine and respiratory tree, respectively (Fig. 7). CAT activity in respiratory tree was significantly higher than in intestine, and in muscle was the lowest among the three tissues \((P<0.05)\). CAT activity in muscle of the regeneration group was significantly higher than in the control during the experiment \((P<0.05)\), which increased after evisceration and the maximum value occurred on the 20th day, then decreased gradually until the end of the experiment. After the 20th day, no significant difference of CAT activity in the regenerated intestine occurred between regeneration and the control groups \((P>0.05)\). CAT activity in the regenerated respiratory tree was significantly lower than that in the control on the 20th day after evisceration \((P<0.01)\), then decreased gradually to the normal level at the end of the experiment.

3.3.5. Total antioxidant capacity

T-AOC value in muscle of \(A. japonicus\) gradually increased after evisceration and was significantly higher than the control from the 10th to 25th days \((P<0.05, Fig. 8)\). After that, it decreased gradually to the level of the control at the end of the experiment \((P>0.05)\). For the regenerated intestine and respiratory tree, T-AOC values were very significantly higher than those in the control on the 20th day after evisceration \((P<0.01)\) then decreased gradually to the normal level of the control at the end of the experiment.

3.3.6. Malonyl-dialdehyde

MDA values in muscle of \(A. japonicus\) were significantly higher than in the control after evisceration \((P<0.01, Fig. 9)\), then fluctuated and gradually decreased to the control level on the 30th day. For the regenerated intestine and respiratory tree, MDA values were significantly higher than those in the control on the 20th day after evisceration \((P<0.01)\), then decreased gradually to the normal level at the end of the experiment \((P>0.05)\).

4. Discussions

Generally, energy source used for animals’ metabolism and growth comes from both endogenous and exogenous substance. Endogenous substance is the energy accumulated in the body, while exogenous substance is the energy obtained from feed (Shearer, 1994). Results of the present study showed that the body weight of sea cucumbers was reduced significantly when they eviscerated viscera and partial coelomic fluid. Since there was no energy intake before the intestine regenerated and digestive function resumed, sea cucumbers in this study only consumed endogenous substance to regenerate its internal organ, recover immune capacity and maintain basic metabolism. Hence after evisceration, sea cucumber body weight was reduced until the 10th day. Similarly, Yuan et al. (2007) reported that sea cucumber body weight was reduced during aestivation due...
to absence of digestive function and consumption of endogenous substance. A previous study reported that it takes about two weeks for A. japonicus to regenerate a new intestine with partial digestive function (Zheng et al., 2006). Likewise sea cucumber in this study regained partial ability of ingestion and digestion on the 15th day and then altered from consuming solely endogenous substance to the combination of endogenous and exogenous substance. In this study, the body weight of a typical sea cucumber increased gradually after the 15th day of evisceration. Although accelerated growth rates were observed in the regeneration group from the 10th day to 20th day, their final body weight did not equal or surpass the control's body weight on the 45th day after evisceration.

Respiratory tree is the primary respiratory organ of the sea cucumber; cutaneous respiration is the other major form of respiration (Choe, 1963). Before regeneration, sea cucumbers obtain oxygen via cutaneous respiration to maintain their energy metabolism. However, as shown in a previous study (Tan et al., 2008), the accessory respiratory organ cannot provide sufficient oxygen intake, and as a result, OCR reduced rapidly after evisceration. Afterwards, sea cucumber OCR increased gradually with new respiratory tree regenerated and partial function resumed. On the 45th day after evisceration, when sea cucumber regenerated their respiratory tree, no significant difference in OCR was found between the regeneration and control group, similar to result from a previous study (Zheng et al., 2006).

The health of animals might be evaluated by sensitive immunomarkers. The ideal immunomarker indicates not only the health of the animals but also the degree of environmental stress on immune systems (Wang et al., 2008b). Susceptibility to disease may increase if immunomarkers are below the normal standard. Evisceration of sea cucumber is a protective behavior to cope with severe environmental challenge, and sea cucumbers enter a regeneration stage after evisceration (Fan et al., 2007; Zheng et al., 2006). Similar to mollusks, sea cucumbers cope with foreign materials via both cellular and humoral mechanisms in which many non-specific, defense-related parameters are involved (Dyrynda et al., 1998; Renault et al., 2001; Wootton and Pipe, 2003). Thus, major immunoenzyme activities including ACP, AKP from hydrolytic system and SOD, CAT, MDA, T-AOC from antioxidant system, of sea cucumber could be monitored to evaluate the immunity status during regeneration (Wang et al., 2008a, 2008b).

ACP is a phosphatase, a type of enzyme, used to free attached phosphate groups from other molecules during digestion (Cajaraville et al., 2000; Rajalakshmi and Mohandas, 2005). AKP is a metalloenzyme, which catalyzes the nonspecific hydrolysis of phosphate monoesters (Zhang et al., 2004). When exposed to a variety of environmental stress, as lysosomal enzymes, ACP and AKP also participate in degradation of foreign proteins, carbohydrates and lipids (Ottaviani, 1984; Pipe et al., 1993; Xue and Renault, 2000). In the present study, evisceration significantly affected activities of hydrolases in sea cucumber. A significant increase of ACP activity in muscle was observed on the 10th day after evisceration, and this is consistent with the increase of AKP activity. The mutual increases in both ACP and AKP activities suggest not only enhancement of the capacity of degradation and defense to foreign materials, but also improvement of metabolic intensity to provide more energy for the regeneration of the viscera in sea cucumber.

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**Fig. 8.** Changes of T-AOC activity in muscle, intestine and respiratory tree of A. japonicus during the regeneration of viscera. Each bar represents means ± SE. Asterisks indicate significant differences with respect to control values: *P*<0.05; **P**<0.01, and NS indicates no significant difference.
As regeneration advanced, ACP and AKP activities in all three tissues returned to normal, and no significant difference was found between the regeneration and control groups on the 45th day after evisceration except for ACP activity in intestine, which was significantly higher than the control probably due to the enhanced digestion in the regenerating intestine.

The adaptation of organisms to environmental stress depends on its antioxidant defense, and those species with lower antioxidant efficiency may be less tolerant of conditions affecting oxidative damage (Regoli et al., 2004). Because oxygen radicals are continuously generated by a variety of cellular processes, all organisms have evolved antioxidant defenses which can be measured by total antioxidant capacity (T-AOC) with both enzymatic and non-enzymatic components. Enzymatic defenses include superoxide dismutase (SOD) and catalase (CAT), which detoxify $O_2^-$ and $H_2O_2$, respectively (Hermes-Lima et al., 1998). MDA is a measure of terminal products of lipid hydroperoxides, which reflects oxyradicals stress on organisms. In the present study, T-AOC value in muscle increased and peaked on the 20th day after evisceration, and those in the regenerated tissues elevated significantly as well. SOD activities in the regenerated tissues significantly enhanced after evisceration until the 45th day. During the whole period of the experiment, CAT activities in muscles of the regeneration group were significantly higher than in the control, which ascended after evisceration and peaked on the 20th day. Correspondingly, a significant increase in MDA value for both muscle and regenerated tissues was observed on the 20th day after evisceration. More interestingly, there is a clear opposite trend in the response of CAT and SOD in the respiratory tree. A low-level but enhancing CAT activity in the regenerating respiratory tree was detected, and returned to normal on the 45th day. Comparatively, SOD activity in the respiratory tree significantly enhanced on the 20th day after evisceration, then decreased gradually, but still significantly higher than that in the control at the end. As we know, SOD catalyzes the conversion of $O_2^-$ into $H_2O_2$, while catalyzes the decomposition of $H_2O_2$ to water and oxygen, which usually are an important antioxidant defense in nearly all cells exposed to oxygen (Hermes-Lima et al., 1998). However, the invertebrate immune system was more than a collection of simple innate responses (H. glaberrima, Ramírez-Gómez et al., 2008). It has been revealed that $H_2O_2$ also can be catalyzed by other peroxidases, such as glutathione peroxidase (GPX; Hermes-Lima et al., 1998). In the present study, the increasing CAT activity indicated that CAT might participated in parts of $H_2O_2$ detoxification in the regenerating respiratory tree, however, if there were any other specific peroxidases involved in the regeneration of the respiratory tree and their possible roles was still unknown, which deserved further research in the future.

The immune response to evisceration and the regeneration of viscera appeared to be an adaptation allowing the sea cucumber to manage oxidative stress during regeneration. With regeneration of the respiration tree, OCR of sea cucumber increased to normality at the 45th day. Thus, the sea cucumber displayed coordinate changes in antioxidant defenses including an increase before the 20th day and the decrease afterwards mitigating oxidative stress that occurred as part of natural cycles of regeneration. With the MDA and T-AOC activities returned to normal on the 45th day, the mission of the antioxidant defenses during regeneration were completed. Unfortunately, the molecular mechanisms that trigger and regulate changes in antioxidant enzyme activities in sea cucumbers are still unknown but could prove to have key relevance to regeneration of new tissues. In the other

Fig. 9. Changes of MDA activity in muscle, intestine and respiratory tree of A. japonicus during the regeneration of viscera. Each bar represents means ± SE. Asterisks indicate significant differences with respect to control values: *$P<0.05$; **$P<0.01$, and NS indicates no significant difference.
animals, the oxidative stress could play an important role in the onset and progress of a reduced capacity for wound healing and tissue regeneration, and an up-regulating of antioxidant enzymes were also detected during this stage in the polychaete *Eurythoe complanata* (Nusatti et al., 2005), which was similar with the present study.

The immune responses of *A. japonicus* to evisceration were tissue-specific and changed in different temporal patterns during the regeneration of the viscera. Results from the present study demonstrated that non-specific immune parameters in muscle significantly enhanced after evisceration generally. However, those in the regenerated intestine and respiratory tree showed different trends. SOD activity, T-AOC and MDA values were significantly higher than the control when measured on the 20th day, while AKP activity in respiratory tree and APK activity in intestine were significantly lower, and no significant difference was found in AKP activity for respiratory tree and CAT for intestine. On the 45th day after evisceration, if taking changes in SGR and OCR into account together, most immune parameters returned in different temporal patterns to normal levels, which might indicate that *A. japonicus* recovered to normal physiology from evisceration.

Previous studies showed that new intestine functions for digestion on the 21st day in *Holothuria glaberrima* (García-Arrarás et al., 1998) and on the 14th day in *A. japonicus* after evisceration (Zheng et al., 2006), respectively. However, at least 35 days are required for the new intestine to return to normal (García-Arrarás et al., 1998; Zheng et al., 2006). Similar to results from Zheng et al. (2006), in the present study, it was observed that new intestine resumed its normal digestive function on the 15th day and indicated that the first two or three weeks after evisceration might be a critical period for the regeneration of the viscera in *A. japonicus*. This may have practical implications for sea cucumber farmers and suggests that management should eliminate possible environmental stressors in the first two to three weeks after evisceration. On the other hand, immunostimulants are suggested added during the regenerating course, such as lipopolysaccharides, as described in *H. glaberrima* (Santiago-Cardona et al., 2003).

In conclusion, the results present valuable data regarding the growth, metabolism and non-specific immune parameters in response to evisceration and regeneration of viscera in *A. japonicus*. Although the eviscerated sea cucumbers did not catch up with the controls in body weight, their SGR, OCR and major non-specific immune parameters resumed to normal levels implying physiological recovery within 45 days after evisceration.

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